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Original Research

Interactive Effects of Copper, Fluorene, and Fluoranthene on Enzymatic Biomarkers and Metallothionein Levels in Crucian Carp (*Carassius auratus*)

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Abstract

To evaluate potential interactive effects of metals and polycyclic aromatic hydrocarbons (PAHs) on biomarker responses, groups of the freshwater fish crucian carp (*Carassius auratus*) were exposed to single and binary combinations of copper (Cu) (0.01-0.16 mg L-1) with fluorene (Fl) or fluoranthene (Fluo) (2-10 mg kg^{-1}) for 96 h. Dose-dependent increases in the activities of phases I and II metabolic enzymes [7-ethoxyresorufin O-deethylase (EROD) and glutathione-S-transferase (GST), respectively] were observed in fish liver exposed to Fl and Fluo, but these enzyme activities did not differ significantly from the controls when co-treated with higher concentrations of Cu, suggesting an inhibiting interaction on the metabolic enzymes. Although Cu did not alter catalase (CAT) activity, CAT activity was decreased in fish liver exposed to the two PAHs alone or in combination with Cu. Although metallothionein (MT) content in gills was significantly increased following exposure to Cu alone or in combination with Fl and Fluo, the induction folds of MT decreased under co-exposure. Co-exposures to these chemicals invoked complex biomarker responses in fish liver and gills. These results highlight the need for careful consideration of the interactive effects of multiple environmental stressors on fish.

Keywords: copper, fluorene, fluoranthene, *carassius auratus*, biomarkers

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Introduction

Generally, inorganic and organic contaminants coexist in aquatic environments. These chemicals in mixtures may interact with aquatic organisms such as fish, and alter the toxicity of each chemical greatly. Metals and polycyclic aromatic hydrocarbons (PAHs) are, respectively, among the most typical inorganic and organic contaminants. Aquatic pollution from metals and PAHs has been recorded for decades. Due to their ubiquitous use, they are still pervasive in the aquatic environment in recent years [1-3]. The detrimental effects of individual metals and PAHs on fish have been substantiated by a number of studies [4-6]. But still there are gaps in our knowledge on the combined toxicity of these pollutants [7], although co-contamination of metals and PAHs has been constantly found in the aquatic environment [8-10].

The modes of toxic action on non-target aquatic organisms between metals and PAHs are quite different. For instance, the metals are able to increase the reactive oxygen species (ROS) generation through redox cycling; the protein content and the catalytic activities of cytochrome P450 (CYP) enzymes can be reduced when ROS act as second messengers, leading to activation of kinases with phosphorylation of CYP proteins or when they interact with the iron of heme groups [11]. In contrast, most PAHs can be biologically oxidized by the CYP enzymes and in turn induce enzymatic activity. Hence, the interactive effects of metals and PAHs toward fish may be complex and, in fact, be far from those predicted in an additive way [7].

Being a biologically essential trace element, copper (Cu) is widespread in aquatic systems [12]. But at elevated concentrations it can be toxic to fish associated with reproductive impairments, larval deformities, reduced growth, and behavioral changes, etc. [6]. For PAHs, previous studies focused mainly on the toxicity of high molecular weight (HMW) PAHs (5- and 6-fused aromatic ring, e.g., benzo[a]pyrene) on fish, because of their high mutagenicity and carcinogenicity. Though being less mutagenic, low molecular weight (LMW) PAHs such as fluorene (Fl, 3-fused aromatic ring PAH) and fluoranthene (Fluo, 4-fused aromatic ring PAH) can represent much more ecological risk than HMW PAHs in the aquatic environment [13]. However, the effects of LMW PAHs on fish are sparsely described, especially in scenarios of co-exposure with metals.

Here, to give a comprehensive account of the effects of binary mixtures of Cu plus Fl or Fluo on crucian carp (*Carassius auratus*), a wide range of biochemical biomarkers in fish tissues were applied in the present study. Phase I and II metabolism enzymes (the activities of 7-ethoxyresorufin O-deethylase (EROD) and glutathione-S-transferase (GST), respectively) and anti-oxidative enzyme (the activity of catalase (CAT)) were evaluated in fish liver. Moreover, as an advisable indicator of metal exposure, change in metallothionein (MT) content was determined in fish gills exposed to Cu^{2+} alone or in combination with PAHs. Through this study, we expect to provide a better understanding of the interactive effects between metals and PAHs on aquatic organisms.

Experimental Procedures

Chemicals

Fl, Fluo, β-Nicotinamide adenine dinucleotide 2'-phosphate-reduced tetrasodium salt (NADPH), 7-ethoxyresorufin, and haemoglobin were purchased from Sigma Chemical (St. Louis, MO, USA) and the stated purity was >98%. CuSO4 (analytical grade) was obtained from Shanghai Tingxin Chemistry Reagent Co., Ltd. (Shanghai, China). 1-chloro-2,4-dinitrobenzene (CDNB) and bovine serum albumin (BSA) were purchased from Shanghai Huixing Biochemistry Reagent Co., Ltd. (Shanghai, China), and the purity was >98%. All other chemicals were of analytical grade and were obtained from Shanghai Chemical Reagent Co., Ltd. (Shanghai, China).

Animals and Exposure

Immature fish were obtained from the Nanjing Institute of Fishery Sciences (Nanjing, China). The mean body weight and length of fish were 25.4±4.6 g and 11.8±1.6 cm, respectively. Before batch tests, the fish were acclimatized in de-chlorinated municipal water ($CaCO_3$ 104.3±6.1 mg L^{-1}, pH 7.1±0.3, 16±2ºC) for two weeks. The fish were fed every day with uncontaminated commercial pellet food (Tetra Pond Sticks, Germany). Sewage and uneaten food were removed every other day by suction. The fish were not fed for 24 h prior to the experiments, and no food was provided during the test period.

Randomly assigned fish were divided into various exposure groups (four fish per treatment) under constant aeration. The fish loading rate ranged 0.7-1.0 g L^{-1}. Cu was added into the water in the solution form of $CuSO_4$, while Fl and Fluo treatments were conducted via intraperitoneal injection after being dissolved in corn oil due to the extremely low solubility in water. Based on the earlier fish toxicological studies concerning the target chemicals [14-15], the nominal exposure concentrations were set as 0.01, 0.02, 0.04, 0.08, and 0.16 mg L^{-1} in the Cu-alone treatments, and the Fl- and Fluo-alone exposure treatments were administrated at dosages of 2, 4, 6, 8, and 10 mg kg^{-1}. In the binary exposure treatments (i.e., Cu+Fl and Cu+Fluo), 10 mg kg^{-1} Fl or Fluo in corn oil was injected into fish, which were kept in the Cu^{2+}-contained water at concentrations ranging from 0.01 to 0.16 mg L^{-1}. Two control treatments were employed: a blank control group and a solvent control group (corn oil). The test water renewal was performed every day. All the treatments were conducted in triplicate. After 96 h of exposure, the fish were sacrificed by cervical transection after being anaesthetized with buffered tricaine methanesulfonate (100 mg L^{-1}). The tissues (liver and gills) were removed

immediately, washed in 0.15 M KCl, weighed, and stored at -80ºC.

Enzyme Assays

Liver samples were homogenized in nine volumes of cold buffer (0.15 M KCl, 0.1 M Tris-HCl, pH 7.4) and then centrifuged for 25 min (10,000×g) at 4ºC. The supernatants were used as the tissue extract for enzymatic activity determination. Liver EROD activity was quantified at 572 nm using 96-well plates as described by [4]. The reaction mixture consisted of 140 µL buffer (0.1 mol L^{-1} Tris, 0.15 mol L^{-1} KCl, pH 8.0), 10 µL of 2 µmol L^{-1} 7-ethoxyresorufin, and 10 µL microsomes. The reaction was initiated at 25°C by the addition of 40 µL of 2.1 mg mL^{-1} NADPH. GST activity was determined at 340 nm by adapting to a microplate reader using 30 µL of homogenate and 150 µL of the reaction solution (100 µL of 0.1 mM potassium phosphate, 10 µL of 1.0 mM 1-chloro-2,4-dinitrobenzene, 10 µL of 1.0 mM GSH, and 880 µL H_2O) [16]. CAT activity was determined by the method of ammonium molybdate [17]. Initially, 200 µL of the homogenate was incubated with 1 mL of substrate (65 mM hydrogen peroxide in 60 mM potassium phosphate buffer, pH 7.4) at 37ºC for 60 s. The enzymatic reaction was terminated by adding 1 mL of 32.4 mM ammonium molybdate and yellow complex of molybdate and hydrogen peroxide was measured at 405 nm.

Gill samples were homogenized in five volumes of cold phosphate buffer 0.1 M (pH 7.2, triton 1%) on ice and centrifuged for 20 min (10,000×g) at 4ºC. The concentration of MT was determined by the Cd-haemoglobin saturation method [18]. Five-hundred microliters of 200 mg L^{-1} $CdCl_2$ solution was added into 200 µL of tissue homogenate and allowed to incubate at room temperature for 15 minutes. Then, 200 µL of 2% bovine haemoglobin solution (w/v) was added to the sample, and the sample was mixed, heated, and centrifuged, after which the supernatant was collected. The Cd concentration in the supernatant was measured using flame atomic absorption equipment (TAS-986, Purkinje General Instrument Co. Ltd., Beijing, China), and the MT concentration was calculated from the Cd concentration measured in the supernatant.

Using the coomassie protein assay kit, protein concentrations were determined at 595 nm as previously described [19], with bovine serum albumin as standard.

Data Analysis

The results are expressed as the mean ±standard deviation (SD, $n = 3$). The data from different treatments within each experiment were compared using two-way analysis of variance (ANOVA), and significant differences were identified by Dunnett's test. A p-value<0.05 was accepted to be statistically significant. Statistical analyses were conducted with SPSS statistical package (ver. 17.0, SPSS Company, Chicago, IL, USA).

Results

No mortality occurred during the 96-h exposure periods. Between the blank controls and the solvent controls, there were no significant differences in the responses of biomarkers in fish. Hence, liver EROD, GST, and CAT activities as well as MT content in gills in fish exposed to target compounds were compared with those in the blank controls. The activity of EROD in the liver tissues after 96 h of exposure to Cu^{2+} and PAHs are presented in Fig. 1. Compared to the control, both PAHs at the lower dosages (2 or 4 mg kg^{-1}) did not change EROD activity obviously (p>0.05); but at the higher dosages, Fl and Fluo significantly increased liver EROD activity to 1.5- and 1.3-fold, respectively (Fig. 1a). In contrast, liver EROD activity was remarkably inhibited by exposure to Cu^{2+} alone at the concentrations of 0.04, 0.08, and 0.16 mg L^{-1}; the maximal inhibition rate obtained at the concentration of 0.16 mg L^{-1} was 30% (Fig. 1b). Compared to the isolated PAH exposures, the addition of Cu^{2+} decreased liver EROD activity in most cases. But both the co-treatments of Cu^{2+} and Fl or Fluo did not significantly alter liver EROD activity compared with that

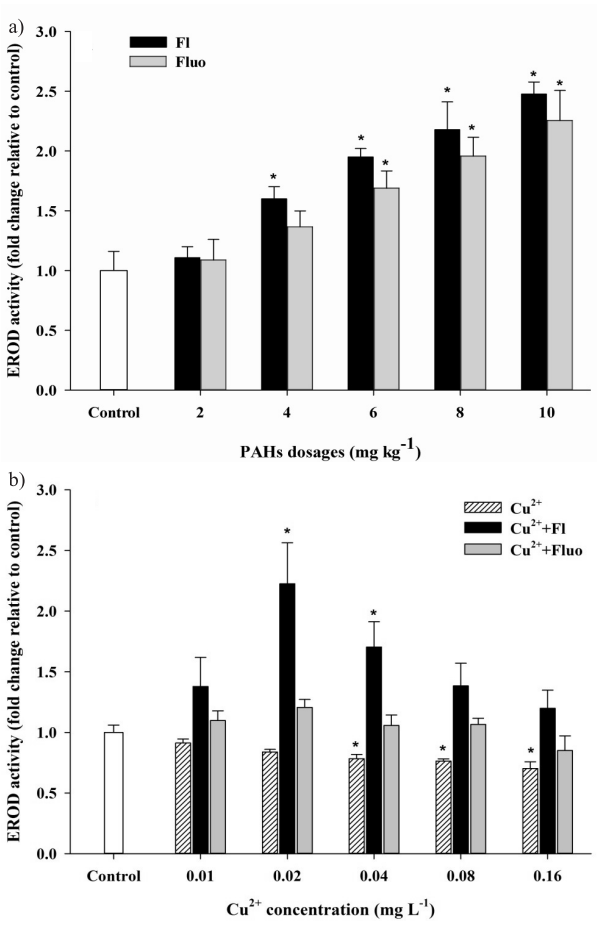

Fig. 1. Responses of liver EROD activity after 96-h exposure to: a) Fl, Fluo and b) Cu^{2+} alone and in combination with 10 mg kg^{-1} Fl or Fluo. Error bars indicate ±SD (n = 3). Asterisks indicate values that are significantly different from control values (p<0.05).

in the control group, except at the Cu^{2+} concentrations of 0.02 and 0.04 mg L^{-1} in the Cu^{2+} and Fl treatments, where the liver EROD activity was acutely induced (1.2- and 0.7-fold, respectively; Fig. 1b).

The activity of GST in the liver tissues after 96 h of exposure to Cu^{2+} and PAHs are presented in Fig. 2. Across all the test dosages, Fl and Fluo significantly increased GST activity (Fig. 2a). Both the Fl and Fluo exposures resulted in the greatest GST increase (2.0- and 1.5-fold, respectively) at the highest dosages of 10 mg kg^{-1}. As shown in Fig. 2b), at the nominal concentrations of 0.01 and 0.02 mg L^{-1}, GST activity was markedly increased (1.1- and 1.6-fold, respectively) by the Cu^{2+}-alone treatment; but at higher concentrations (0.04, 0.08, and 0.16 mg L^{-1}), the GST activity did not show obvious differences from the control. Compared to the control, the liver GST activity was not significantly changed by the co-treatment of Cu^{2+} plus Fl (p>0.05). However, the binary exposure of Cu^{2+} at concentrations of 0.01, 0.02, and 0.04 mg L^{-1} plus Fluo did increase the liver GST activity with the maximal induction of 1.6-fold.

The activity of CAT in the liver tissues after 96 h of exposure to Cu^{2+} and PAHs are presented in Fig. 3. Fl- and Fluo-alone exposures decreased liver CAT activity at all the dosages (Fig. 3a). The two highest dosages (8 and 10 mg kg^{-1}) of PAHs significantly inhibited CAT activity with the maximal inhibition rate of 46% by Fl exposure and 62% by Fluo exposure. As shown in Fig. 3b), when compared to the control, no obvious difference in CAT activity was observed in fish liver exposed to Cu^{2+} alone (p>0.05). In fish liver exposed to the mixtures of Cu^{2+} and Fl, CAT activity was markedly inhibited (inhibition rate of 36%) only at the Cu^{2+} concentration of 0.16 mg L^{-1}, while the combination of Cu^{2+} and Fluo obviously suppressed CAT activity in most cases with the most significant inhibition rate of 50% (Fig. 3b).

The MT content in the gills after 96 h of exposure to Cu^{2+} and PAHs are presented in Fig. 4. As shown in Fig. 4a), MT content in fish gills was not obviously affected by the PAH-alone treatments at all the test dosages (p>0.05). But a concentration-dependent increase of MT content was observed in Cu^{2+}-alone treatment, reaching the maximal fold change (1.2-fold) at the highest exposure concentration (Fig. 4b). Similarly, MT content in the gills was distinctly increased by the combinations of Cu^{2+}+Fl and Cu^{2+}+Fluo (with the greatest increases of 0.9- and 0.8-fold, respectively) in most cases, although slightly lower than that in the Cu^{2+}-only counterparts (Fig. 4b).

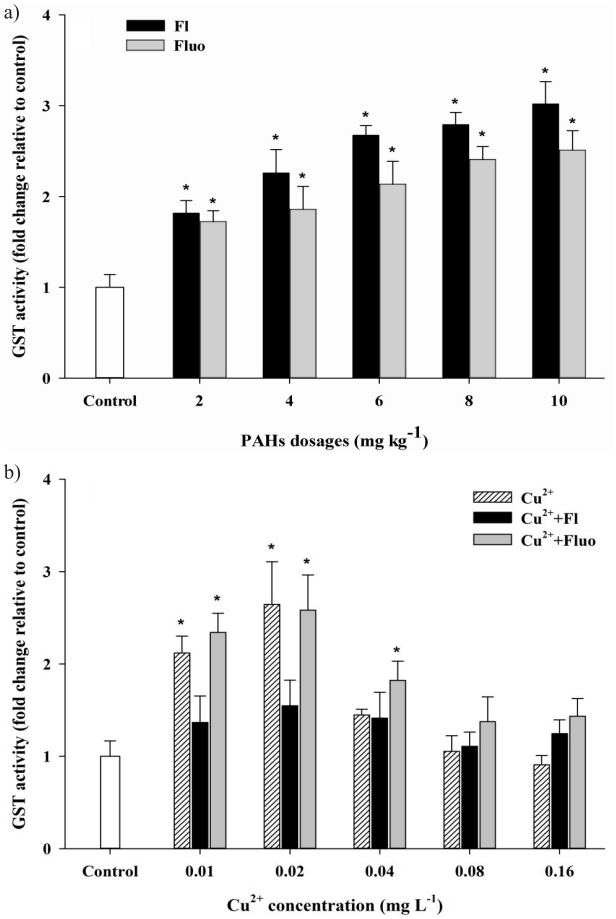

Fig. 2. Responses of liver GST activity after 96-h exposure to a) Fl, Fluo and b) Cu^{2+} alone and in combination with 10 mg kg^{-1} Fl or Fluo. Error bars indicate ±SD (n = 3). Asterisks indicate values that are significantly different from control values (p<0.05).

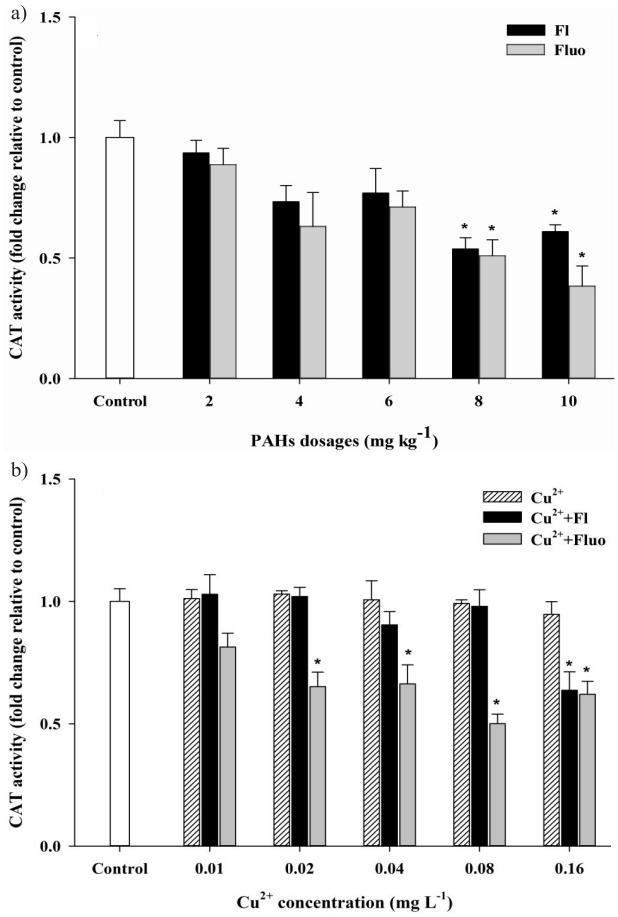

Fig. 3. Responses of liver CAT activity after 96-h exposure to a) Fl, Fluo and b) Cu^{2+} alone and in combination with 10 mg kg^{-1} Fl or Fluo. Error bars indicate ±SD (n = 3). Asterisks indicate values that are significantly different from control values (p<0.05).

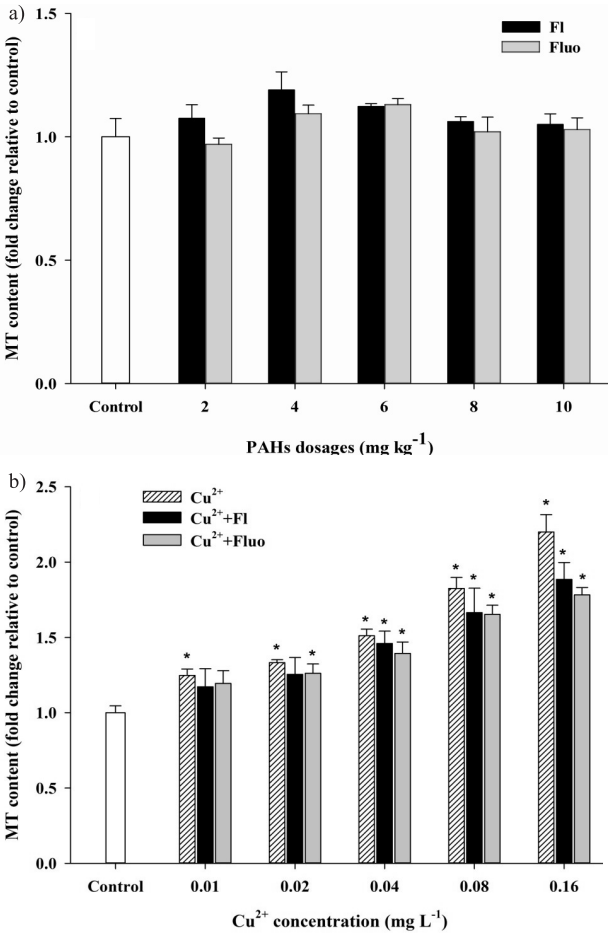

Fig. 4. Responses of gill MT content after 96-h exposure to a) Fl, Fluo and b) Cu^{2+} alone and in combination with 10 mg kg^{-1} Fl or Fluo. Error bars indicate ±SD (n = 3). Asterisks indicate values that are significantly different from control values (p<0.05).

Discussion of Results

In general, xenobiotics are metabolized in fish through a two-step process involving a biotransformation step with CYP enzymes (phase I) accompanied by a conjugation step with GST (phase II). Associated with CYP1A, EROD can catalyze phase I metabolism of many chemicals. Hence, numerous fish studies used this enzyme as a biomarker for potential phase I metabolism. In the present study, liver EROD activity was increased by the isolated Fl and Fluo exposures at the higher dosages, which was in agreement with a previous finding in *C. auratus* [4]. Conversely, linking the inability to bind to Ah-receptor (AhR) and initiate transcription [15, 20], unapparent change or even inhibition of EROD activity by Fl and Fluo exposures has been reported in *in vivo* studies with other fish species, including zebrafish (*Danio rerio*) [21], gilthead seabream (*Sparus aurata*) [22], and mummichog (*Fundulus heteroclitus*) [15]. These results suggest that, unlike most HMW PAHs, the biochemical response to LMW PAHs may not be uniform among different fish species. Due to the great diversity found in some 28,000 fish species [23], there could be large intrinsic variations in the affinity of the AhR of different fish species to small molecular-size PAH compounds [22, 24]. Unlike Fl and Fluo, single exposure to Cu^{2+} inhibited the liver EROD activity in *C. auratus* in this study, as also found in other fish species exposed to Cu^{2+} [25-27]. When co-treated with Fl and Fluo, the addition of Cu^{2+} also led to a decrease in EROD induction, compared respectively to that in the fish treated only with 10 mg kg^{-1} Fl and Fluo. Similarly, reduction by zinc(II) in benzo(*k*)fluoranthene-increased EROD activity has been observed in the *C. auratus* hepatocytes [28]. Inhibition in liver EROD activity may be due to the divalent metal ions that can bind to the sulfhydryl group, leading to a protein conformational change and consequently disrupting the normal function of the enzyme [27, 29-30]. Thus, in field sites with multi-pollution of environmental stressors, detection of PAH pollution by EROD activity might be masked by the simultaneous presence of such metals [31].

After biotransformation, metabolites will experience conjugation process and eventually convert into more water-soluble forms to be excreted from the body, which is usually related to catalysis by GST. Moreover, by metabolizing lipid hydroperoxides in response to ROS production, GST plays a critical role in the antioxidant defense system [32]. In this study, the marked increase of GST activity was observed in the fish liver exposed to Fl and Fluo alone. The increased GST activity by Fl or Fluo was also recorded in the same species [4, 33] and different species, such as crab (*Carcinus maenas*) [34]. These results suggest the involvement of GST in the detoxification of these two PAHs and the occurrence of oxidative stress response in aquatic organisms to Fl and Fluo exposure. GST activity in liver was significantly increased at the lower concentrations of Cu^{2+} (≤0.02 mg L^{-1}) but began to decrease at the higher concentrations. Considering that the EROD activity was inhibited by Cu^{2+} in the present study, the increase in GST activity in the fish liver exposed to lower concentrations of Cu^{2+} could be related to enhanced ROS production, in accordance with the hypothesis that Cu^{2+} would directly cause oxidative stress via Fenton reaction [25], while the unchanged GST activity at the higher Cu^{2+} concentrations might be due to the fact that excess Cu^{2+} causes rapid glutathione (GSH) oxidation, and also reduces the GSH/oxidized glutathione (GSSG) ratio in hepatocytes, followed by GST depletion [35-36]. In regards to binary co-exposure groups, the fish exposed to Cu^{2+} plus Fl or Fluo did not show any obvious changes in liver GST activity compared to the control in most cases, suggesting that the addition of Cu^{2+} has reduced the induction of GST activity caused by Fl or Fluo exposure. Lack of effects on GST activity may also be attributed to the variation of GSH/GSSG ratio, as co-exposure of Cu^{2+} and PAHs would lead to stronger GSH oxidation. Collectively, the interaction between metals and PAHs could elicit inhibiting effects on EROD and GST activities in fish liver compared to those in the isolated treatments. The result indicates that coexistence of metals and PAHs may hamper the activation of fish metabolic enzymes, and eventually result in the bioaccumulation risks of these contaminants.

In addition to GST, CAT is also an extensively expressed antioxidant enzyme in fish. Located in the peroxisomes, CAT is able to catalyze the decomposition of hydrogen peroxide to oxygen and water [37]. The available data in fish studies, which concerns the response of CAT activity on LMW PAHs exposure, is not consistent when some demonstrate stimulation in CAT activity [38-39] while others report no change or even significant inhibition in this enzyme activity [22]. In our study, fish liver CAT activity obviously decreased by isolated exposure to Fl and Fluo (8 and 10 mg kg^{-1}), suggesting that these two PAHs at high doses may inhibit CAT activity and thus suppress the scavenging capacity of fish to ROS. Inhibited CAT activity together with elevated GST activity indicate a perturbation of antioxidant enzyme systems and potential oxidative damage in the fish hepatocytes caused by Fl and Fluo. The lack of response of CAT activity on Cu^{2+} exposure was observed in the liver of *C. auratus* in this study, and other fish species including tilapia (*Oreochromis niloticus*) [40], catfish (*Rhamdia quelen*) [25], and neotropical fish (*Prochilodus lineatus*) [41], indicating that CAT activity in fish may not be a sensitive biomarker for Cu pollution. For the binary exposure of Cu^{2+} and Fl, the liver CAT activity was still unchanged in most cases. However, the activity of this enzyme was significantly inhibited by exposure to the mixture of Cu^{2+} and Fluo in this study. This result demonstrated that the antioxidant system of *C. auratus* may fail to scavenge overproduced ROS by co-exposure to Cu^{2+} and Fluo.

Owing to the wide surface area in contact with water, gills are an important uptake route of organisms for waterborne pollutants [42]. Among metal ligands, induced MTs are involved in non-enzymatic homeostasis and a detoxification mechanism of fish against metal exposure. In previous studies, the induction of MT content in fish gills exposed to metals has been documented in tilapia (*Oreochromis mossambicus*) [43-44], common carp (*Cyprinus carpio*) [45-46], and catfish (*Clarias gariepinus*) [47]. Our study also demonstrated that waterborne exposure to Cu^{2+} could increase MT contents in gills of *C. auratus* in a concentration-dependent manner, confirming that MT synthesis level in *C. auratus* gills is a sensitive biomarker for aquatic pollution of metals. Although Fl- and Fluo-alone exposures did not obviously change the MT content in gills, joint exposures of Cu^{2+} and Fl or Fluo still enabled the increase of MT levels in most cases in the present study. But it should be noted that co-administrations with Fl and Fluo slightly decreased the MT synthesis when compared to exposure to Cu^{2+} alone. The down-regulation of metal-induced MT levels in fish by PAHs has been found in some earlier studies, and this observation may be due to competition for cysteine residues by GSH synthesis [28, 48]. Moreover, this indicates that co-exposure with PAHs may suppress elimination of Cu ions, and thus enhance accumulation and toxicity of Cu^{2+} in fish.

Conclusions

This study shows various biochemical effects in crucian carp (*C. auratus*) exposed to the metal Cu ion, the PAHs Fl and Fluo, and their binary mixtures. After 96 h of exposure, liver EROD and GST activities seemed not to be obviously affected by the co-exposure of Cu^{2+} combined with Fl or Fluo. This suggests that these two enzymes in *C. auratus* may be of limited use in the ecological risk assessment of multi-pollution by metals and PAHs. However, liver CAT activity was significantly inhibited by Cu^{2+} and Fluo exposure, and MT content in gills was highly active to both the co-treatments of Cu^{2+} and Fl or Fluo. These results indicate that the joint exposure of metals and PAHs could trigger more severe accumulation risks and oxidative stress in *C. auratus*. Despite these results, further study is required to understand deeply and to develop more appropriate biomarkers of the interaction with metals and PAHs in fish.

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